JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1987, p. 242–247
0095-1137/87/020242-06$02.00/0

Vol. 25, No. 2

Detection of Immunoglobulin G to *Pasteurella haemolytica* Capsular Polysaccharide by Enzyme-Linked Immunosorbent Assay

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Received 5 June 1986/Accepted 14 October 1986

An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of immunoglobulin G (IgG) to the capsular polysaccharide (CP) of *Pasteurella haemolytica* serotype 1. Purified CP was first covalently coupled to poly-L-lysine and then optimally adsorbed at a concentration of 5 μg/ml to microtiter plates in the presence of carbonate-bicarbonate buffer (pH 9.8). The ELISA was used to evaluate and compare the CP-specific IgG response of calves vaccinated with different *P. haemolytica*-derived experimental vaccines. Elevated levels of ELISA IgG titers were detected in postvaccination sera and lung lavage from calves vaccinated intradermally with live logarithmic-phase organisms or the culture supernatants. The ELISA was found to be a rapid, reproducible, and sensitive technique for the detection of CP-specific antibodies and may be useful to delineate the protective role of these antibodies in bovine pneumonic pasteurellosis.

Pasteurella haemolytica serotype 1 is widely recognized as the agent responsible for severe fibrinous pneumonia in pneumonic pasteurellosis (shipping fever) of cattle (2, 3). The polysaccharide nature of the bacterial capsule has been established (7), and immunological differences among capsular polysaccharide (CP are the basis for division of *P. haemolytica* into 15 serotypes (5). The capsule is produced in large amounts by in vitro-propagated logarithmic-phase cells (11). Capsulated organisms have been shown to be more severe pathogens to cattle than noncapsulated organisms (R. E. Corstvet and R. J. Panciera, Annu. Meet. Conf. Res. Workers Anim. Dis. 1980, abstr. no. 92, p. 17).

Antigenic materials that contained a mixture of CP, lipopolysaccharide, and outer membrane proteins have been extracted from *P. haemolytica* serotype 1 and used successfully as vaccines in experimental trials (9, 15, 20, 23, 34). However, in none of these studies was it possible to determine a protective role for antibody to CP because assays for the measurement of this antibody were unavailable. Therefore, it appears that a need exists for a widely accessible, sensitive, and reproducible assay for the detection and quantification of antibody to *P. haemolytica* serotype 1 CP.

We used a recently described method for purification of CP (1) and then developed an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin G (IgG) in bovine sera and lung washings to this purified polysaccharide. The results demonstrate that this assay is highly sensitive, specific, and reproducible.

MATERIALS AND METHODS

Preparation of CP. *P. haemolytica* 12296 was isolated in pure culture from the lungs of a yearling feedlot calf that died of pneumonic pasteurellosis. It was identified as serotype 1 by the rapid plate agglutination procedure (12) and was maintained in the lyophilized state. The organism was grown on the surface of sterile polycarbonate membrane (0.2-μm pore size; Nuclepore Corp., Pleasonton, Calif.) which was overlaid on solid Sawata medium (19). The plates were incubated at 37°C for 6 h under a 10% CO_2 atmosphere. The bacteria were suspended in phosphate-buffered saline (PBS, pH 7.4) at 56°C for 1 h in a shaking water bath and removed by centrifugation at 13,000 × g for 20 min.

The supernatant containing the crude capsular extract (14) was filter sterilized, dialyzed for 48 h, and lyophilized. Capsule removal was confirmed by the Maneval method of staining (22). The CP was purified by the procedure of Adlam et al. (1). Briefly, the crude capsular extract was first subjected to methanol precipitation to remove protein contaminants. The lipopolysaccharide was removed by phenol extraction and ultracentrifugation. The CP was then precipitated with ethanol and purified by molecular sieve column chromatography. The purified polymer (designated CP) contained approximately 95% hexosamine (28) and was free of protein (25) and lipopolysaccharide (17, 18). Nuclear magnetic resonance spectroscopy of the CP showed the compound to be relatively clear of proteinaceous material with a spectrum similar to that previously described (1).

Coupling of CP to poly-L-lysine. Purified CP was covalently bound to 0.1% poly-L-lysine (60,000 molecular weight; Sigma Chemical Co., St. Louis, Mo.), using cyanuric acid as the coupling agent (16). This was a three-step procedure. First, the CP was alkalinized, followed by activation and slow acidification of the CP by cyanuric chloride. The final step involved the covalent coupling of the CP to poly-L-lysine.

ELISA reagents. Carbonate-bicarbonate buffer (pH 9.8) was used as the coating buffer. The washing solution was deionized water containing 0.05% Brij 35 (Sigma). The sample diluent was PBS (pH 7.4) containing 0.05% Brij 35 and 0.3% bovine serum albumin (PBS-Brij-BSA). The conjugate was horseradish peroxidase-conjugated rabbit anti-bovine IgG (H- and L-chain specific; Miles Laboratories, Inc., Naperville, Ill.) diluted in PBS–0.05% Brij without BSA. The substrate solution was 2,2-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (Sigma). No preservatives were used.

ELISA procedure. The procedure described by Voller et. al. (30) for microtiter plate indirect ELISA was adapted with some modifications. Checkerboard titrations were used to determine the most effective coating buffer, antigen concen-

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tration, optimal conjugate dilution, choice of a diluent, and optimal incubation period.

In subsequent tests, individual wells of 96-well polyvinyl U-bottomed microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va) were first coated with 100 μl of a 5-μg/ml solution of CP-coupled antigen diluted in coating buffer. The plates were coated overnight at room temperature in a reciprocating shaker. The plates were washed three times with washing solution, sealed, and stored at 4°C. Before use, 100 μl of diluent buffer was added to each well, incubated for 1 h at 37°C, and suctioned dry. A 100-μl sample of the appropriate dilution of the test samples in PBS-Brij-BSA was added, and the plates were incubated at 37°C for 2 h. The plates were then washed three times, and a 100-μl sample of conjugate diluted 1:4,000 was added to each well. The plates were incubated for 1 h at 37°C and then washed six times. A 100-μl portion of substrate was added, and the plates were incubated at 37°C until proper color development occurred, which was a preestablished absorbance reading of the known reference positive serum. Incubation time was 10 to 20 min, and the reaction was not stopped chemically before reading the absorbance. Color development was measured on dual wavelengths, 405 nm test and 490 nm reference, with a Dynatech MR-580 spectrophotometer. Controls included were the antibody control, which contained all the reagents except the antigen, and the antigen control, which contained all the reagents except antibody. Appropriate substrate and poly-L-lysine controls were also tested for nonspecific color development.

Sera and lung lavage samples collected from two colostrum-deprived (CD) calves which did not contain any antibodies to the surface determinants of *P. haemolytica* were used as standard negative controls in the ELISA test. Serum and lung lavage samples collected from a CD calf immunized twice with a crude capsular extract from *P. haemolytica* served as reference positive control. Immunization was accomplished intramuscularly with 5 mg of capsular extract emulsified in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), followed by a booster 3 weeks later with extract emulsified in Freund incomplete adjuvant (Difco).

Study groups. Sera and lung lavage samples from calves used in our different vaccination trials were analyzed by the ELISA for the presence of antibody to CP of *P. haemolytica*.

In experiment A, 13 calves were acquired within 4 h after birth and given one feeding of colostrum obtained from their dams at the farm. The quantity of colostrum fed was 6 lb (2.7 kg) per 100-lb (45-kg) weight of calf. Thereafter, the calves were transported and raised in isolation rooms for 9 to 11 weeks (colostrum-fed [CF] calves). Calves maintained by this protocol had low colostrum-derived antibody to *P. haemolytica* (21) in their sera and were free from nasal colonization with *P. haemolytica* (data not shown). At this time, calves were randomly separated into three groups. Six calves in group 1 were vaccinated intradermally (i.d.) at multiple sites in the neck with 1 ml of 10^9 CFU of live log-phase *P. haemolytica* 12296 per ml propagated in RPMI 1640 medium (4) (live bacteria vaccinates). Group 2 calves consisted of three calves vaccinated i.d. with 1 ml of culture supernatant from a log-phase bacterial growth (culture supernatant vaccinates). This supernatant contained a mixture of CP, leukotoxin, lipopolysaccharide, and proteins. Four calves in the control group were injected i.d. with 1 ml of RPMI 1640 medium (sham vaccinates).

In experiment B, two CD calves were raised inside isolation rooms for 9 weeks and then vaccinated as described above for production of positive control sera (culture supernatant + adjuvant vaccinates). The culture supernatant used for vaccination was partially purified by ultrafiltration on a Diaflo XM300 membrane (Amicon Corp., Lexington, Mass.) and then lyophilized.

Experiment C consisted of two CD calves and three CF calves vaccinated ID at 1 week of age with 1 ml of live log-phase bacteria containing 10^9 CFU/ml.

Blood was collected from all calves from experiments A, B, and C before vaccination (prevaccination) and weekly up to 7 weeks after vaccination (postvaccination). All sera were heat inactivated, filter sterilized, and stored at −80°C.

Lung lavage was performed on all calves, except those from experiment C, at prevaccination and postvaccination by the procedure described by Wilkie and Markham (31). The lavage samples were filtered through cotton gauze and centrifuged at 200 × *g* for 4 min to remove cells and then centrifuged at 40,000 × *g* for 30 min at 4°C to remove lung surfactant. The resulting lavage fluid was dialyzed against distilled water and stored in the lyophilized state until it was analyzed. For the ELISA, lung lavage samples were reconstituted in PBS-Brij-BSA diluent to contain 1,200 μg of protein per ml (25). Serial dilutions were then made in the diluent, and 100 μl was used in the ELISA.

All test and control samples were run in triplicate, and each experiment was performed at least three times.

Expression of results and statistics. The ELISA endpoint (30) titer of each test sample was designated as the reciprocal of the serum or lung lavage dilution which gave an optical density (OD) of 0.1. This was calculated from a titration curve graph which plotted OD as function of serum or lung lavage dilution.

Collective titers for groups of calves from experiments A, B, or C were expressed as ELISA geometric mean titer (ELISA GMT). The GMT (8) gives equal weight to all test values and is not skewed toward extreme values.

Linear regression analysis (29) was used to establish a regression line on the linear portion of each ELISA titration curve. The slope values for these regression lines were tabulated, and a mean slope value for each vaccine group was then calculated. To answer the question whether the mean slope values correlated with ELISA endpoint titers, we calculated the correlation coefficients or *r* values. The associated *P* values were determined from a table of *r* values.

The coefficient of variation (29), a measurement of the amount of variation in grouped data, was used to analyze the precision and reproducibility of the ELISA tests.

The Student *t* test (29) for paired comparisons was used to analyze the significance of difference between the logarithm of the prevaccination and postvaccination ELISA and endpoint titers.

A one-way analysis of variance (29) was used to compare the ratio of postvaccination to prevaccination ELISA endpoint titers. From the analysis of variance sum of squares, an a priori test was used to calculate the *P* values of the differences between vaccinate groups.

RESULTS

Determination of optimal conditions for ELISA. A series of experiments were performed to study various factors having a possible influence on the ELISA. Initial experiments evaluated two coating buffers on the adsorption of poly-L-lysine-coupled CP to the microtiter plates. Plates coated

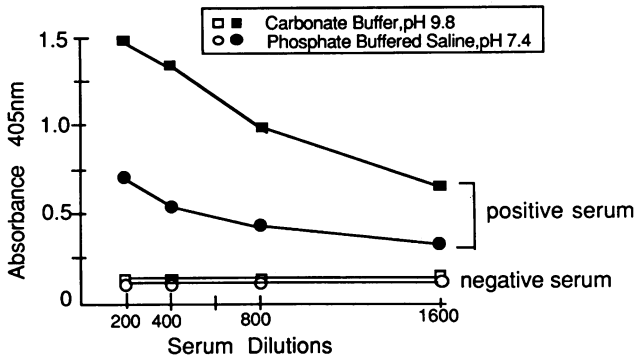

FIG. 1. Comparison of coating buffers on ELISA sensitivity. Microtiter plates were coated with 7.5 μg of poly-L-lysine-coupled *P. haemolytica* CP antigen per ml contained in the different coating buffers. The standard negative serum (prevaccination) and positive serum (postvaccination) were diluted in PBS-BRIJ-BSA. A 1:4,000 dilution of horseradish peroxidase-conjugated anti-bovine IgG was used. The data points depict means of four separate experiments, each performed in triplicate. The figure shows that carbonate-bicarbonate buffer at pH 9.8 was best suited for use as antigen coating buffer.

with the carbonate buffer resulted in optimal adsorption over plates coated with PBS buffer (Fig. 1). In subsequent experiments carbonate buffer was used as the coating buffer.

Optimal concentrations of antigen and conjugate were determined by checkerboard titration with reference positive and negative standards. The optimal antigen concentration was 5 μg/ml with a conjugate dilution of 1:4,000 (Fig. 2).

Incubation times of less than 1 h were tested in conjunction with temperatures above 37°C in an attempt to reduce the total assay time below 3 h. The elevated temperatures caused a significant increase in color development above maximum limits in all the wells including the negative control.

We found that preincubation of the antigen-coated plates with the diluent PBS-Brij-BSA for 1 h at 37°C and using this buffer as the sample diluent minimized a high nonspecific background adsorbance due to poly-L-lysine binding to the conjugate. A 2-h incubation for the antigen-antibody step gave the greatest differentiation between the reference negative and positive sera or lung lavage samples.

Precision and reproducibility of ELISA. Reproducibility of the assay was studied by running 10 triplicate determinations of a single positive serum sample. When performed on the same day with the same preparation of antigen, conjugate, and substrate, the coefficient of variation of OD readings was 4.1 to 11.3%. When performed on different days the variation was 7.4%. Plate-to-plate variation was 10%.

Using numerous separate assays, we calculated a standard OD value for the positive reference serum. If during sample assays the OD value of the positive reference serum showed more than 11% variation from the previously determined standard OD value, a correction ratio was calculated. The sample OD values were then corrected by dividing the OD of each sample by the correction ratio. Besides triplicate wells of the known positive dilution and known negative dilution, the positive serum serially diluted was run on each plate so that a standard curve and slope for corrections (6, 27) could be used if necessary.

Application of ELISA for detection of IgG against *P. haemolytica* CP in vaccinated calves. ELISA titers were determined for sera and lung lavage samples collected from all calves in experiments A, B, and C. Results from experiments A and B (Fig. 3 and 4) compare ELISA GMT at prevaccination and 5 weeks postvaccination for serum samples and 4 weeks postvaccination for lung lavage samples. Since inadequate amounts of lung lavage samples from 5 weeks postvaccination were available, we selected the 4 week postvaccination lung lavage data for illustration in Fig. 4.

Prevaccination sera from both CF and CD calves from experiments A and B had low levels of IgG to CP as shown by the ELISA GMT with a range from 350 to 1,800. Similarly, lung lavage from prevaccination calves also showed a low ELISA GMT with a range from 25 to 230.

In experiment A sham-vaccinated calves, the *t* test showed no significant difference in the ELISA endpoint titers between pre- and postvaccination sera and lung lavages ($P > 0.20$). In contrast, sera from live bacteria vaccinates showed a significant rise in postvaccination ELISA endpoint titers ($P = 0.044$) and also a significant rise in lung lavage titers ($P = 0.038$). Sera from culture supernatant vaccinates showed a rise in postvaccination titers ($P = 0.182$), and lung lavage titers showed no significant rise ($P > 0.20$).

A strong correlation with a 95 to 99% certainty was observed between the mean slope values and mean ELISA endpoint titers of prevaccination and all 2, 3, 4, and 5 week

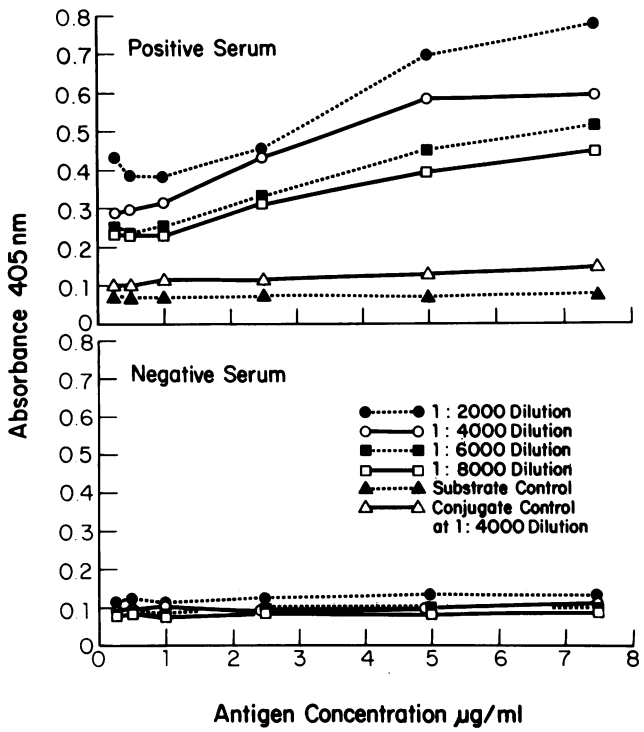

FIG. 2. Results of the checkerboard titration showing determination of optimal amounts of *P. haemolytica* CP antigen (coupled to poly-L-lysine) and horseradish peroxidase-antibovine IgG conjugate required for the ELISA test. Included on the plates were the substrate and conjugate controls. Various dilutions of conjugate and various concentrations of CP antigen were tested with both positive and negative sera. Negative and positive sera (similar to Fig. 1) were used at a 1:800 dilution. The data points are means of four separate experiments, each performed in triplicate. To conserve reagents, a conjugate dulution of 1:4,000 and an antigen concentration of 5 μg/ml were considered best.

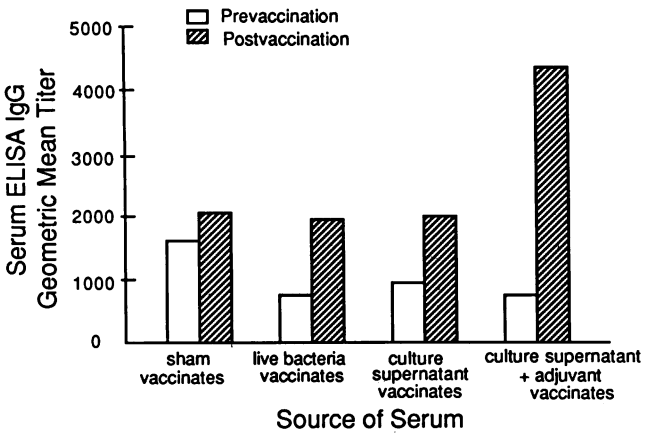

FIG. 3. Comparison of IgG to *P. haemolytica* antigen measured by ELISA in the sera of vaccinated and sham-vaccinated calves from experiments A and B. Results from the three groups in experiment A are depicted on the left side of the figure, while the group from experiment B is on the far right. Postvaccination ELISA titers were from serum samples collected 5 weeks after vaccination. Each bar represents the ELISA GMT.

postvaccination sera and lung lavage samples from live bacteria and culture supernatant vaccinates in experiment A. In contrast, a poor correlation was observed with samples from the sham vaccinates.

The one-way analysis of variance test on the ratios from the three groups in experiment A showed a significant difference among these experimental groups ($P < 0.01$) for both serum and lung lavage ratios. Further comparison of the groups indicated a significant difference between the sham vaccinates and the live bacteria vaccinates ($P = 0.017$ for serum and $P = 0.043$ for lung lavage samples). No significant difference was detected between the sham vaccinates and culture supernatant vaccinates in both serum and lung lavage ($P > 0.30$).

The Student *t* test used on the results from experiment B calves showed differences that were not statistically significant ($P = 0.157$ for serum, $P = 0.089$ for lung lavage), although a large difference was seen between the pre- and postvaccination ELISA GMT of sera and lung lavage (Fig. 3 and 4). This disparity is due to the low numbers of calves and therefore a degree of freedom of only 1.

In an unrelated experiment with CF calves from experiment A (data not shown), we found that analysis of serum samples collected from neonatal calves (precolostral) and 24 h after colostrum feeding (postcolostral) suggested that there was passive transfer of CP-specific IgG through colostrum. To determine whether preexisting colostrum-derived antibody against *P. haemolytica* CP interfered with antibody response to vaccination, we immunized a group of 1-week-old CD and CF calves (experiment C) i.d. with a live vaccine. Sera collected at prevaccination and 2, and 3 weeks postvaccination were analyzed by ELISA for the presence of CP-specific antibody. Whereas immunization of CD calves induced a big rise in antibody titer, there was no rise in the antibody titer in the sera from CF calves (Fig. 5). The rise in CP-specific ELISA endpoint titers in CD calves at 3 weeks had a meaningful difference in the paired *t* test ($P = 0.071$), and those of CF calves had no significance ($P > 0.50$). The *t* test on the corresponding slope values of CD calves showed statistical significance ($P = 0.049$), and the slope values of CF calves had no significance ($P > 0.50$).

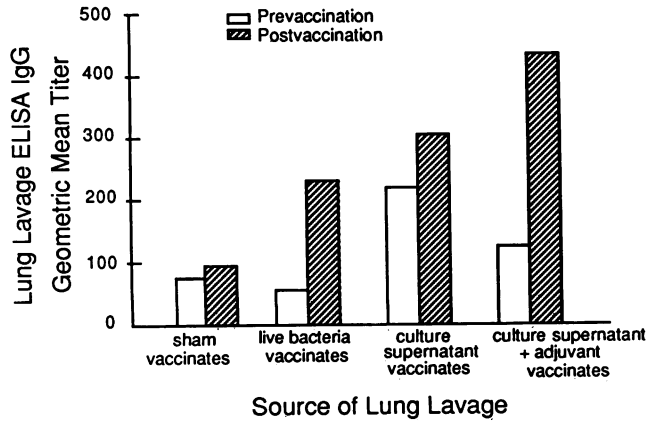

FIG. 4. Comparison of IgG to *P. haemolytica* CP antigen measured by ELISA in the lung lavage of vaccinated and sham-vaccinated calves from experiments A and B. Postvaccination ELISA titers were from lung lavage samples obtained 4 weeks after vaccination. ELISA titers and expressed as described in the legend to Fig. 3.

DISCUSSION

The ELISA methodology developed in this study is well suited for detection of the antibody response to CP of *P. haemolytica* type 1 in bovine sera and lung lavage.

We used both endpoint titers and slope values to evaluate this response. In general, the ELISA endpoint titers of serum and lung lavage samples correlated fairly well with the slope values. However, looking at both endpoint titers and slope values may give added insight to the results, especially if critical evaluation is needed.

In experiment C the slope values gave a more significant value than did the endpoint titers. The negative or poor correlation of endpoint titers to slope values in control or sham-vaccinated calves in experiment A is expected since no new antibody should be generated. Slope values that do not increase in conjunction with increasing endpoint titers probably indicate that a high-affinity antibody is not being produced. Other researchers have also indicated that slope values may permit measurement of antibody affinity to the antigen and that high-affinity antibody may be more protective (6).

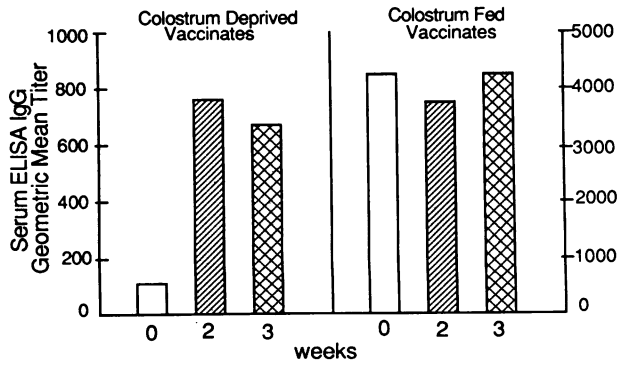

FIG. 5. IgG to *P. haemolytica* antigen detected by ELISA in prevaccination (▭), 2 weeks postvaccination (▨), and 3 weeks postvaccination (▩) sera obtained from calves in experiment C vaccinated i.d. with live logarithmic-phase bacteria. ELISA titers were expressed as described in the legend to Fig. 3.

Our studies showed that i.d. vaccination of 9- to 11-week-old CF calves (which had low levels of colostrum-derived CP-specific antibody at prevaccination) with live log-phase bacteria resulted in a significant increase in the CP-specific IgG titers in the sera and lung lavage (Fig. 3 and 4). However, vaccination of 1-week-old CF calves (which had high levels of colostrum-derived CP-specific antibody at prevaccination) with live bacteria did not induce elevated antibody titers to CP (Fig. 5). A possible explanation of this phenomenon was only speculative. It was quite possible that the passively acquired (via colostrum) CP-specific IgG in the sera inhibited the immune response to active immunization. These findings are similar to those of others (31, 32) who showed that systemic and lung humoral response to vaccination was inversely related to the magnitude of antibody titers found in these secretions before vaccination. The finding that adjuvant-treated culture supernatant derived from a log-phase bacterial culture induced a marked increase in the CP-specific ELISA IgG titer in the lung lavage (Fig. 4) was a very exciting finding. The significance of this finding can only be evaluated if large numbers of calves are used.

During the development of this procedure, several problems were encountered. The most significant problem was that the purified CP adsorbed very poorly to the solid phase of the ELISA microtiter plates. We found that it was first necessary to covalently bind the CP to poly-L-lysine. Optimal adsorption was accomplished in the presence of carbonate-bicarbonate buffer at pH 9.8. Others (16, 24) have shown that the function of the poly-L-lysine is to adsorb to the solid phase, thus immobilizing the coupled polysaccharide. We also discovered that the adsorbed poly-L-lysine can bind directly with the conjugate and give a high background adsorption reading. These high background readings were drastically reduced by using BSA in the diluent and adding a presample incubation step.

Good reproducible results were obtained by our ELISA method with CP-bound poly-L-lysine. The antigen-coated plates could be stored at 4°C for several months without losing antigenic activity (data not shown). It showed high specificity because we used purified CP as the antigen. Furthermore, the method was sensitive enough to demonstrate small changes in antibody levels.

So far, no methodology is available to measure the protection-inducing capacity of *P. haemolytica* CP. Recent studies have demonstrated that immunization of cattle with live logarithmic-phase *P. haemolytica* by the parenteral or aerosol route can effectively protect cattle from experimental pasteurellosis (10, 26). It has been speculated that the possible mechanisms which log-phase organisms provide better protection than stationary-phase organisms are related to the amount of leukotoxin and capsule (4, 11). Gentry et al. (13) suggested that resistance to disease correlated directly with antileukotoxin titers. Since no serological assay was available to detect CP-specific antibody, its role in protection has not been studied.

Bovine pneumonic pasteurellosis is an economically important disease in beef and dairy cattle in the United States (33). A test suitable for quantitation of IgG antibody against the CP of type 1 *P. haemolytica* will define its role in immunity to this disease. We demonstrated that capsule-specific IgG can be detected in the lung lavage of calves vaccinated with live log-phase *P. haemolytica* organisms or their culture supernatant. Clearly, further work has to be done to establish a correlation between anticapsular antibody concentration in the bovine lung, the isotype measured by ELISA, and protection against pneumonic pasteurellosis.

ACKNOWLEDGMENTS

This work was supported in part by special grant 85-CRSR-2-2629 from the U.S. Department of Agriculture.

We thank John Anderson, Department of Biochemistry, University of Minnesota, for his valuable assistance and kindly performing the nuclear magnetic resonance spectroscopy analysis.

LITERATURE CITED

1. **Adlam, C., J. M. Knights, A. Mugridge, J. C. Lindon, P. R. W. Baker, J. E. Beasley, B. Spacey, G. R. Craig, and L. K. Nagy.** 1984. Purification, characterization and immunological properties of serotype-specific capsular polysaccharide of *Pasteurella haemolytica* (serotype A-1) organisms. J. Gen. Microbiol. **130:** 2415–2426.
2. **Allen, E. M., H. A. Gibbs, A. Wiseman, and I. E. Selman.** 1985. Sequential lesions of experimental bovine pneumonic pasteurellosis. Vet. Rec. **117:**438–442.
3. **Ames, T. R., R. J. F. Markham, J. Opuda-Asibo, J. R. Leininger, and S. K. Maheswaran.** 1985. Pulmonary response to intratracheal challenge with *Pasteurella haemolytica* and *Pasteurella multocida*. Can J. Comp. Med. **49:**395–400.
4. **Baluyut, C. S., R. R. Simonson, W. J. Bemrick, and S. K. Maheswaran.** 1981. Interaction of *Pasteurella haemolytica* with bovine neutrophils: identification and partial characterization of a cytotoxin. Am. J. Vet. Res. **42:**1920–1926.
5. **Biberstein, E. L.** 1978. Biotyping and serotyping of *Pasteurella haemolytica*. Methods in Microbiol. **10:**255–269.
6. **Carlson, B. A., G. S. Giebink, J. S. Spika, and E. D. Gray.** 1982. Measurement of immunoglobulin G and M antibodies to type 3 pneumococcal capsular polysaccharide by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **16:**63–69.
7. **Carter, G. R.** 1956. A serological study of *Pasteurella haemolytica*. Can. J. Microbiol. **2:**483–488.
8. **Colton, T.** 1974. Statistics in medicine, 1st ed., p. 30. Little, Brown, & Co., Boston.
9. **Confer, A. W., B. A. Lessley, R. J. Panciera, R. W. Fulton, and J. A. Kreps.** 1985. Serum antibodies to antigens derived from a saline extract of *Pasteurella haemolytica*: correlation with resistance to experimental bovine pneumonic pasteurellosis. Vet. Immunol. Immunopathol. **10:**265–278.
10. **Confer, A. W., R. J. Panciera, R. E. Corstvet, J. A. Rummage, and R. W. Fulton.** 1984. Bovine pneumonic pasteurellosis: effect of culture age of *Pasteurella haemolytica* used as a live vaccine. Am. J. Vet. Res. **45:**2543–2545.
11. **Corstvet, R. E., M. J. Gentry, P. R. Newman, J. A. Rummage, and A. W. Confer.** 1982. Demonstration of age-dependent capsular material on *Pasteurella haemolytica* serotype 1. J. Clin. Microbiol. **6:**1123–1126.
12. **Frank, G. H., and G. E. Wessman.** 1978. Rapid plate agglutination procedure for serotyping *Pasteurella haemolytica*. J. Clin. Microbiol. **7:**142–145.
13. **Gentry, M. J., A. W. Confer, and R. J. Panciera.** 1985. Serum neutralization of cytotoxin from *Pasteurella haemolytica*, serotype 1 and resistance to experimental bovine pneumonic pasteurellosis. Vet. Immunol. Immunopathol. **9:**239–250.
14. **Gentry, M. J., R. E. Corstvet, and R. J. Panciera.** 1982. Extraction of capsular material from *Pasteurella haemolytica*. Am. J. Vet. Res. **43:**2070–2073.
15. **Gilmour, N. J. L., W. B. Martin, J. M. Sharp, D. A. Thompson, and P. W. Wells.** 1979. The development of vaccines against pneumonic pasteurellosis in sheep. Vet. Rec. **104:**15.
16. **Gray, B. M.** 1979. ELISA methodology for polysaccharide antigens: protein coupling of polysaccharides for adsorption to plastic tubes. J. Immunol. Methods **28:**187–192.
17. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
18. **Keleti, G. W., and H. Lederer.** 1974. Handbook of micromethods for the biological sciences, p. 74–75. Van Nostrand Reinhold Co., New York.

19. **Kume, K., A. Sawata, and Y. Nakase.** 1978. Haemophilus infections in chickens. I. Characterization of *Haemophilus paragalinarum* isolated from chickens affected with coryza. Jpn. J. Vet. Sci. **40**:65–73.
20. **Lessley, B. A., A. W. Confer, D. A. Mosier, M. J. Gentry, J. A. Durham, and J. A. Rummage.** 1985. Saline-extracted antigens of *Pasteurella haemolytica*: separation by chromatofocusing, preliminary characterization, and evaluation of immunogenicity. Vet. Immunol. Immunopathol. **10**:279–296.
21. **MacDonald, J. T., S. K. Maheswaran, J. Opuda-Ashibo, E. Townsend, and E. S. Thies.** 1983. Susceptibility of *Pasteurella haemolytica* to the bactericidal effects of serum, nasal secretions and bronchoalveolar washings from cattle. Vet. Microbiol. **8**:585–599.
22. **Maneval, W. E.** 1941. Staining bacteria and yeasts with acid dyes. Stain Technol. **16**:13–19.
23. **Matsumota, M., J. A. Schmitz, B. Syuto, B. J. Watrons, and D. E. Mattson.** 1984. Immunogenicity of a soluble antigen against *Pasteurella haemolytica*-associated pneumonia in calves. Vet. Res. Commun. **8**:117–130.
24. **Messina, J. P., P. G. Hickox, M. L. Leprow, B. Pollara, and R. A. Venezia.** 1985. Modification of a direct enzyme-linked immunosorbent assay for the detection of immunoglobulin G and M antibodies to pneumococcal capsular polysaccharide. J. Clin. Microbiol. **21**:390–394.
25. **Miller, G. L.** 1959. Protein determination for large numbers of samples. Anal. Chem. **31**:964.
26. **Panciera, R. J., R. E. Corstvet, A. W. Confer, and C. N. Gresham.** 1984. Bovine pneumonic pasteurellosis: effect of vaccination with live *Pasteurella* species. Am. J. Vet. Res. **45**: 2538–2542.
27. **Pederson, F. K., and J. Henricksen.** 1982. Detection of antibodies to pneumococcal capsular polysaccharides by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **15**:372–378.
28. **Smith, R. L., and E. Gilkerson.** 1979. Quantitation of glycosaminoglycan hexosamine using 3-methyl-2-benzothiazone hydrazone hydrochloride. Anal. Biochem. **98**:478–480.
29. **Sokal, R. R., and F. J. Rohlf.** 1969. Biometry—the principles and practice of statistics in biological research, p. 204–234, 424, 515–523. W. H. Freeman & Co., San Francisco.
30. **Voller, A., D. E. Bidwell, and A. Bartlett.** 1979. The enzyme-linked immunosorbent assay (ELISA). A guide with abstracts of microplate applications, p. 18–40. Dynatech Laboratories, Inc., Alexandria, Va.
31. **Wilkie, B. N., and R. J. F. Markham.** 1979. Sequential titration of bovine lung and serum antibodies after parenteral or pulmonary inoculation with *Pasteurella haemolytica*. Am. J. Vet. Res. **40**:1690–1693.
32. **Wilkie, B. N., R. J. F. Markham, and P. E. Shewen.** 1980. Response of calves to lung challenge exposure with *Pasteurella haemolytica* after parenteral or pulmonary immunization. Am. J. Vet. Res. **41**:1773–1778.
33. **Yates, W. D. G.** 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can. J. Comp. Med. **46**:225–263.
34. **Yates, W. D. G., P. H. G. Stockdale, L. A. Babiuk, and R. J. Smith.** 1983. Prevention of experimental bovine pneumonic pasteurellosis with an extract of *Pasteurella haemolytica*. Can. J. Comp. Med. **47**:250–256.